PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12Q 1/68, 1/70

A1

(11) International Publication Number: **WO 95/14109**

(43) International Publication Date: 26 May 1995 (26.05.95)

(21) International Application Number: PCT/US94/13514

(22) International Filing Date: 18 November 1994 (18.11.94)

(30) Priority Data:
08/154,416 19 November 1993 (19.11.93) US

(71) Applicant: U.S. DEPARTMENT OF THE ARMY [US/US]; Medical Research and Development Command, Fort Detrick, MD 21702 (US).

(72) Inventors: VAHEY, Maryanne, T.; 19315 Dimona Drive, Brookeville, MD 20833 (US). MICHAEL, Nelson, L.; 17401 Winter Laurel Drive, Olney, MD 20832 (US).

(74) Agents: BENT, Stephen, A. et al.; Foley & Lardner, Suite 500, 3000 K Street, N.W., Washington, DC 20007-5109 (US).

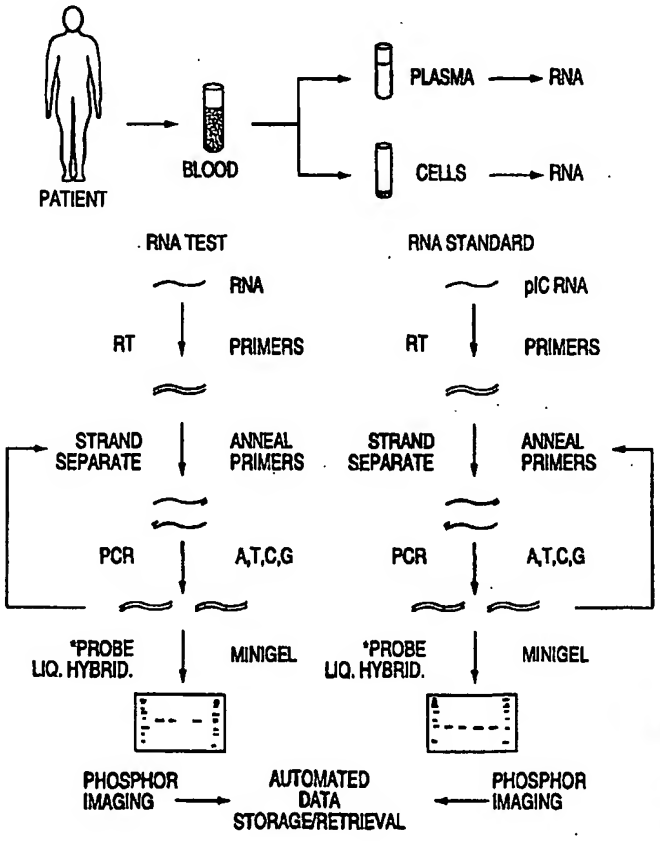
(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LT, LU, LV, MD, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: HIGH THROUGH-PUT QUANTITATIVE POLYMERASE CHAIN REACTION FOR HIV CLINICAL SPECIMENS

(57) Abstract

Methods and kits for the rapid and accurate quantitation of HIV-1 nucleic acids in large numbers of human clinical samples is disclosed. Cloned, standard internal controls for both HIV-1 and cellular sequences are used for parallel amplification and compared with the test samples. A universal HIV-1 *gag* primer permits detection and quantitation of sequences from every known HIV-1 *gag* gene while liquid hybridization detection greatly reduces processing time and error.



FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**GB**	United Kingdom	**MR**	Mauritania
AU	Australia	**GE**	Georgia	**MW**	Malawi
BB	Barbados	**GN**	Guinea	**NE**	Niger
BE	Belgium	**GR**	Greece	**NL**	Netherlands
BF	Burkina Faso	**HU**	Hungary	**NO**	Norway
BG	Bulgaria	**IE**	Ireland	**NZ**	New Zealand
BJ	Benin	**IT**	Italy	**PL**	Poland
BR	Brazil	**JP**	Japan	**PT**	Portugal
BY	Belarus	**KE**	Kenya	**RO**	Romania
CA	Canada	**KG**	Kyrgystan	**RU**	Russian Federation
CF	Central African Republic	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CG	Congo			**SE**	Sweden
CH	Switzerland	**KR**	Republic of Korea	**SI**	Slovenia
CI	Côte d'Ivoire	**KZ**	Kazakhstan	**SK**	Slovakia
CM	Cameroon	**LI**	Liechtenstein	**SN**	Senegal
CN	China	**LK**	Sri Lanka	**TD**	Chad
CS	Czechoslovakia	**LU**	Luxembourg	**TG**	Togo
CZ	Czech Republic	**LV**	Latvia	**TJ**	Tajikistan
DE	Germany	**MC**	Monaco	**TT**	Trinidad and Tobago
DK	Denmark	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**US**	United States of America
FI	Finland	**ML**	Mali	**UZ**	Uzbekistan
FR	France	**MN**	Mongolia	**VN**	Viet Nam
GA	Gabon				

HIGH THROUGH-PUT QUANTITATIVE POLYMERASE CHAIN REACTION FOR HIV CLINICAL SPECIMENS

BACKGROUND OF THE INVENTION

The advent of the polymerase chain reaction, or PCR as it is now known, has had a monumental impact on molecular biology. This powerful technique permits even trace amounts of nucleic acids to be amplified by factors of several logs, depending on the conditions and the number of reaction cycles, thus providing a source of material for experiments that can only be performed when significant quantities of nucleic acid are available.

One of the significant shortcomings of early PCR techniques, however, was an inability to provide quantitative results. Basic PCR can tell if there is a target nucleic acid in a sample and it can amplify that target dramatically, but it cannot accurately determine how much target is present prior to amplification. In the infancy of PCR, this deficiency seemed of minor import as the primary benefit of PCR lay in the identification and exponential increase of nucleic acids. Subsequently, as the full range of possible applications for PCR was realized, those in the field began to fully comprehend the value of an assay like PCR that could also determine levels of nucleic acids within biological samples. Therefore, one of the major goals for researchers over the past several years has been to convert PCR from a qualitative or semi-quantitative method into a truly quantitative assay.

The first attempts at quantitative PCR involved only relative determination of nucleic acids in biological samples. See Kashani-Sabet, *Cancer Res.* **48**:5775-5778 (1988). Other strategies involved the coamplification of endogenous, unrelated sequences with primers different than those used for the targets. See Chelly *et al.*, *Nature* **333**:858-860 (1988); Rappolee *et al.*, *Science* **241**:708-712 (1988). Because even minor differences in the efficiency of the PCR reaction can lead to dramatic

differences in the quantity of distinct nucleic acid products, the ability of this approach to accurately determine absolute starting levels of nucleic acids is limited.

One of the first *bona fide* efforts to achieve true quantitative PCR was reported by Wang *et al.*, *Proc. Nat'l Acad. Sci. USA* 86:9717-9721 (1989). These researchers reported not only relative quantitation but they also allegedly achieved absolute quantitation. Their method of quantitation relies on the use of a standard DNA template that hybridizes to the same, radioactively-labelled primer used for amplification of the test sequences. The standard is added to the test sample and coamplified. Coamplification raises concerns, however, that the cloned template will interfere with the amplification of the test DNA.

Labelling of primers also has a detrimental effect on their hybridization to target sequences. In addition, the use of labeled primers involves detection of the product by hybridization and apparent molecular weight. This method cannot assure that the detected product was generated by amplification of the target sequences, as would be the case where a probe distinct from the primer is used. This is particularly important where HIV-1 is concerned, given the amount of heterogeneity observed in the virus.

Another approach taken by researchers in the quest for quantitative PCR involves the evaluation of PCR kinetics. For example, Noonan *et al.*, *Proc. Nat'l Acad. Sci. USA* 87:7160-7164 (1990), described quantitative analysis of multidrug resistance in human tumors by polymerase chain reaction. Unlike the Wang, Noonan used endogenous nucleic acids that are unrelated to the target for controls. The improvement involves using optimized PCR chemistries that give true exponential amplification for both target and control primers. Using data from the exponential range of amplification, more accurate quantitation was achieved.

More recently, Higuchi *et al.*, *BIO/TECHNOLOGY* **11**:1026-1030 (1993), have reported another approach that provides quantitative results with PCR. Their method involves "real-time" monitoring of increasing ethidium bromide fluorescence in a sample during amplification. A kinetic analysis was performed on this data and, by comparing the normalized fluorescence profiles with standard profiles, an accurate quantitation of the starting material is suggested. This methodology, however, is most useful in examining the dynamics of nucleic acid levels and generally is ill-suited to rapid processing of large number of samples representing a single time point.

Michael *et al.*, *J. Virol.* **66**:310-316 (1992), reported on the evaluation of cryopreserved peripheral blood mononuclear cells from 31 HIV-1 infected patients. These data indicated a correlation of the level of viral DNA and mRNA expression with the stage of HIV-1 infection in humans. To make this determination, the authors developed *gag*-based quantitative PCR and reverse transcriptase (RT)-PCR assays substantively different from any of those reported previously. Specialized internal control plasmids called "pIC's" (plasmid, internal control) were developed that, in essence, mimic the *gag* target sequences under examination. Unlike the methods discussed previously, the cloned standards are used in parallel reactions and are not mixed with the test sample, thus obviating concerns that the standards will interfere with amplification of the test nucleic acid.

Two important results flowed from the selection of the *gag* region as the target. First, *gag* has been identified as a highly conserved region of the HIV-1 genome and, therefore, *gag*-based primers were believed to provide the best chance for hybridization with varying strains of HIV-1. Unexpectedly, however, every single strain of HIV-1 tested to date hybridizes to these primers. Second, the *gag* region resides at the 3'-end of

the viral transcripts. Thus, *gag* is found only in full length products and not in various incomplete replication products. By targeting *gag*, such incomplete transcripts, which are of questionable biological relevance are excluded from the quantitation.

In addition to generating the pIC's for HIV-1 sequences, Michael *et al.* made other pIC's that allowed the quality of cellular DNA and RNA in the samples to be evaluated. In addition, these cellular pIC's, derived from human ß-actin and ß-globin sequences, functioning in the same manner as the HIV-1 pIC's, permit the assessment of viral burden on a "per cell" basis. A further benefit of using external cloned controls is the unlimited supply and unchanging nature thereof. Thus, the Michael *et al.* reported, for the first time, a method for the accurate and invariant quantitation of widely disparate HIV-1 sequences based on cell equivalents and also provided the ability to determine the quality of test nucleic acids.

A major limitation of the assay disclosed by Michael *et al.* is the time-intensive nucleic acid extraction technique. While the standard technique employed yielded representative levels of nucleic acid, it was time consuming, taking about four days on average, required expensive equipment and could only be performed on a limited number of samples, about a dozen, at one time. Another serious limitation was the filter-based Southern blot detection step. Not only is this approach time consuming (three days), but the numerous handling steps increase the chances for erroneous or uninterpretable results.

With the rising incidence of persons afflicted with acquired immunodeficiency syndrome, it has become clear that more efficient methods for accurately diagnosing and monitoring HIV infection are needed. Such methods should maintain the quantitative capabilities achieved with the techniques described above, but also should permit rapid evaluation of large numbers of samples. In addition, the

assay must be "user friendly" and the cost per sample must be relatively low.

In attempting to generate a high throughput assay for detection of HIV-1, the main emphasis has been on achieving a plate-based system. Such systems rely on "capture" technology followed by a colorimetric or chemilluminescent readout. The dynamic range of these assays is low, on the order of 1-2 logs and, therefore, many different reactions must be run on a given sample. Moreover, this kind of assay generally has a threshold detection level of between 10^3 and 10^4 HIV-1 genomes per sample. In addition, the capacity for information handling of plate-based assays is, at the moment, quite limited.

Regardless of the degree to which quantitation of PCR may have been achieved in any of the preceding assays, they are all fairly characterized as either (i) "research and development" methods or (ii) "first generation" high throughput whose technical limitations negate any increased capacity for processing large numbers of samples. In other words, the accuracy needed for quantitative results requires too many control reactions, costly equipment and excessive time commitments. To the extent that large scale, high throughput analysis of clinical samples has been achieved, sensitivity has been unreasonably compromised. Thus, despite the attention that quantitative PCR has achieved, it is clear that the currently available strategies for high throughput assays are all hampered by systems constraints not readily resolved by the existing technology.

SUMMARY OF THE INVENTION

Based on the foregoing, it is clear that a need exists for a quantitative PCR-based assay that is capable of rapidly processing a large number of clinical samples and accurately detecting low levels of HIV-1 sequences. Thus, it is an object of the present invention to provide such an assay for both pure PCR and RT-PCT.

It is also an object of the present invention to provide a kit for quantitative PCR- and RT-PCR-based assays that are capable of rapidly processing a large number of clinical samples and accurately detecting low levels of HIV-1 sequences.

In accordance with foregoing objectives, there is provided a method for the quantitative detection of an HIV DNA in a tissue sample, comprising the steps of:

(i) extracting said HIV DNA from said sample;

(ii) contacting said HIV DNA with

(a) at least four different nucleotide triphosphates,

(b) a first primer that hybridizes to the *gag* region of said HIV DNA, and

(c) an enzyme with polynucleotide synthetic activity,

under conditions suitable for the hybridization and extension of said first primer by said enzyme, whereby a first DNA product is synthesized with said HIV DNA as a template therefor, such that a duplex molecule is formed;

(iii) denaturing said duplex to release said first DNA product from said HIV DNA;

(iv) contacting said first DNA product with a reaction mixture comprising

(a) at least four different nucleotide triphosphates,

(b) a second primer that hybridizes to said first DNA product, and

(c) an enzyme with polynucleotide synthetic activity,

under conditions suitable for the hybridization and extension of said second primer by said enzyme, whereby a second DNA product is synthesized with said first DNA product as a template therefor, such that a duplex is formed;

(v) denaturing said second DNA product from said first DNA product;

(vi) repeating steps ii-v for a sufficient number of times to achieve linear production of said first and second DNA products;

(vii) contacting said first and second DNA products with a first oligonucleotide probe that is labeled and that hybridizes to said first and second DNA products, wherein said contacting is performed in a liquid environment under conditions suitable for hybridization;

(viii) detecting hybridization of said first probe with said first and second DNA products;

(ix) concurrently performing steps ii-viii upon a known amount of a *gag* control DNA in a separate reaction; and

(x) comparing the amount of first and second DNA products generated from said DNA with the amount of products generated from said *gag* control DNA, whereby the amount of HIV DNA in said sample is quantitated.

In another embodiment, there is provided a method for the quantitative detection of an HIV RNA in a tissue sample, comprising the steps of:

(i) extracting said HIV RNA from said sample;

(ii) contacting said HIV RNA with

(a) at least four different nucleotide triphosphates,

(b) a sample reverse transcriptase primer that hybridizes to said HIV RNA, and

(c) an enzyme with reverse transcriptase activity,

under conditions suitable for the hybridization and extension of said sample reverse transcriptase primer by said enzyme, whereby a complementary DNA product is synthesized, such that a duplex is formed.

(iii) contacting said cDNA with

(a) at least four different nucleotide triphosphates,

(b) a first primer that hybridizes to said RNA, and

(c) an enzyme with polynucleotide synthetic activity,

under conditions suitable for the hybridization and extension of said first primer by said enzyme, whereby a first DNA product is synthesized with said cDNA as a template therefor, such that a duplex molecule is formed;

(iv) denaturing said duplex to release said first DNA product from said cDNA;

(v) contacting said first DNA product with a reaction mixture comprising

(a) at least four different nucleotide triphosphates,

(b) a second primer that hybridizes to said first DNA product, and

(c) an enzyme with polynucleotide synthetic activity,

under conditions suitable for the hybridization and extension of said second primer by said enzyme, whereby a second DNA product is synthesized with said first DNA product as a template therefor, such that a duplex is formed;

(vi) denaturing said second DNA product from said first DNA product;

(vii) repeating steps ii-v for a sufficient number of times to achieve linear production of said first and second DNA products;

(viii) contacting said first and second DNA products with a first oligonucleotide probe that is labeled and that hybridizes to said first and second DNA products, wherein said contacting is performed in a liquid environment under conditions suitable for hybridization;

(ix) detecting hybridization of said first probe with said first and second DNA products;

(x) concurrently performing steps ii-ix upon a known amount of a *gag* control DNA in a separate reaction; and

(xi) comparing the amount of first and second DNA products generated from said cDNA with the amount of products generated from said *gag* control DNA,

whereby the amount of HIV RNA in said sample is quantitated.

In another embodiment, there is provided a kit for the quantitative detection of an HIV nucleic acid in a tissue sample, comprising

(i) control DNA's containing HIV *gag* and human ß-globin nucleotide sequences;

(ii) primers derived from HIV *gag*, human ß-globin and human ß-actin nucleotide sequences;

(iii) oligonucleotide probes that hybridize to HIV-1 *gag*, human ß-globin and human ß-actin nucleotide sequences bounded by and exclusive of the corresponding primers of ii; and

(iv) a liquid hybridization chamber containing hybridization buffer.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1. This figure provides a schematic representation of one embodiment of the present invention. RNA is isolated from either plasma or blood cells of a patient. Reverse transcription and PCR

reactions are performed, thereby generating PCR products. These products are reacted with labeled probes in liquid hybridization, separated on a minigel, and the specifically bound probe detected by phosphor imaging. Data is archived and processed by computer.

FIGURE 2. This figure provides a schematic representation of one embodiment of the present invention. DNA is isolated from blood cells of a patient. PCR reaction is performed, thereby generating PCR products. These products are reacted with labeled probes in liquid hybridization, separated on a minigel, and the specifically bound probe detected by phosphor imaging. Data is archived and processed by computer.

DETAILED DESCRIPTION OF THE INVENTION

One of the applications in which high throughput, quantitative PCR would be most helpful is in the diagnosis and treatment of human immunodeficiency virus type 1 (HIV-1) infection. The accurate determination of viral burden and expression in humans is essential for understanding the natural history of HIV-1 disease, predicting disease progression and assessing the efficacy of antiviral intervention strategies. The paucity of expressed viral components in natural infection has made the assessment of viral burden by standard techniques difficult. Viral antigen detection is a poor measure of disease progression. Quantitative viral culture studies have shown a direct correlation with disease progression but these studies are influenced by the strain-selective nature of *in vitro* cocultivation systems.

As described above, Michael *et al.* (1992), employed a unique control system to establish the ability of PCR and RT-PCR quantitatively to assess stages of HIV infection using standardized extraction techniques and traditional Southern blotting. To arrive at an assay that was capable of handling a high number of clinical samples with a high degree of precision at a reasonable cost, it was necessary to overhaul completely this technique, which was based on essentially conventional

reactions. Primarily, consideration was given to (i) reducing the amount of patient material required, (ii) shortening turn around time, (iii) reducing handling steps and, therefore, the opportunities for user error, (iv) increasing the number of samples processed at one time and (v) improving the capacity for rapid readout and data management. Achievement of these goals cannot, however, come at the expense of the sensitivity, dynamic range, accuracy and reproducibility needed in such an assay.

The present invention involves a high throughput assay for the quantitative determination of levels of HIV-1 nucleic acids in clinical samples. Briefly, using improved extraction methods, nucleic acids (RNA or DNA) are isolated from cells in the test sample or, in the case of an acellular sample like plasma, from virions present in the sample. Following extraction, PCR is conducted on the extracted nucleic acid and compared to parallel reactions run with highly characterized cloned standards. As mentioned, the test primers are based on *gag* region sequences that have been found in all reported HIV-1 genomes. When the nucleic acid target is RNA, a rapid reverse transcriptase conversion of RNA to cDNA is performed.

After amplification of the target and control nucleic acids, a rapid liquid hybridization is performed using a *gag*-related probe that hybridizes to a region internal to the amplified sequences. Following separation of the hybridization products, the amount of bound probe can be determined immediately using, for example, a laser-stimulated, phosphorescent imaging system.

Other specialized primer/probe combinations have been designed to serve as controls for sample quantity and quality. These oligonucleotides are derived from ß-globin sequences and human ß-actin and are used (i) to determine the number of cells from which the HIV-1 target DNA or RNA was isolated and (ii) to provide an estimate of the quality of the isolated RNA.

The method can be conducted on up to 500 samples per week with a high degree of accuracy. The start-to-finish time for a single series of samples is 8 hours for DNA and 12 hours for RNA. The instruments need are a thermocycler, mini-gel apparatus and power supply and phosphorescent imaging system, all relatively moderately priced items. Subsequent to start-up investment, the cost per sample is approximately $11.50 per DNA sample and $13.25 per RNA sample, not including personnel.

This assay is amenable to use with tissues, serum, plasma and cells. Approximately, the assay requires RNA purified from 50 μl of plasma. For cell-based assays, about 1-5 X 10^6 cells worth of DNA or RNA are necessary. These amounts are highly conservative when compared with the amount of material necessary for other assays, which routinely require up to ten-fold more cells.

As mentioned, one of the shortcomings in standard PCR and RT-PCR techniques is the lengthy extraction of nucleic acids. The assays used in the present invention employ a two hour scheme for the extraction of nucleic acids from cells and a three hour extraction from virions microfuge pelleted from plasma. While the plasma method was developed for the purposes of this assay, the cell extraction techniques are those provide with the commercial test kit RNA-DNA STAT. Tel-Test B, Inc., Friendswood, TX. The ability to use these more rapid techniques with clinical samples could only be determined empirically. Fortunately, these rapid techniques also were found to be of high efficiency, about 92% (optical density (OD) of 0.74/0.80) for both, as compared with 15-50% for other techniques (OD of 0.15/0.80 for proteinase K digestion; OD of 0.40/0.80 for crude lysis).

As described above, internal plasmic controls or pIC's provide the unique capacity to accurately determine the amount of nucleic acid in a sample. The basic concept is that the pIC mimics the target sequence, in this case the either a *gag* sequence of HIV-1 or ß-globin sequences. By comparing the parallel amplification of a

target nucleic acid with that of a pIC, one can determine the amount of target in the sample. The other benefits of such a pIC-based scheme are (i) avoidance of coamplification effects on the target amplification, (ii) inexhaustive supply of control sequences, (iii) unchanging control sequences and, therefore, unchanging reaction conditions. Essential to a quantitative assessment of sample nucleic acids was the empirical determination of optimal reaction conditions for each of the controls. Only after considerable trial and error with numerous controls were acceptable sequences and conditions identified.

The sequence requirements for pIC's according to the present invention are as follows. The *gag* pIC should contain HIV-1 sequences corresponding to nucleotides 1412 through 1547 of the MN strain. ß-globin constructs comprise nucleotides -195 through +73 of the human ß-globin gene of HUMMBB5E. (ß-actin sequences relevant to primer and probe requirements are derived from nucleotides 1854 through 2170 of the human ß-actin gene of HUMACCYBB). All preceding nucleotide #'s reference the indicated GENBANK sequences. Primers must be derived from these regions with the additional criteria set forth in the following paragraph. Probes for the detection of PCR products must be derived from sequences downstream of the 3'-terminus of the sense primer but upstream of the 5'-terminus of the binding site for the antisense primer.

HIV-specific primers for RT and PCR were selected (i) to amplify only a biologically relevant species and (ii) to hybridize to a target region known to be conserved in geographically diverse samples. For *gag*, primers were selected because they will only amplify full length transcripts. Thus, amplified products are not generated from incomplete transcription products. Primers for both the RT and PCR steps may be generated by any of the standard methods of oligonucleotide synthesis and are then gel purified.

Similarly, the cellular primers specific for ß-globin and ß-actin were selected with particular parameters in mind. First, it is essential that these primers hybridize to a invariant region of the genomes, *i.e.*, exhibit little or no sequence heterogeneity. Second, with respect to RNA, the region of interest also should be ubiquitous. Thus, ß-actin provides an ideal target as actin transcripts are found in almost every cell type.

In reverse transcriptase reactions according to the present invention, the reverse transcriptase used must be free of RNase H activity that is present in some commercial enzymes. In addition, the transcriptases must generate full length transcripts representative of the template. Acceptable reverse transcriptases include those sold by Stratagene, La Jolla, CA (Cat. Nos. 600083 and 600084), Promega, Madison, WI (Cat. Nos. M5301 and M5302), Boehringer Mannheim (Cat. No. 1062 603), Perkin Elmer (Cat No. N808-0017) and Gibco BRL, Gaithersburg, MD (Cat. No. 18053-017). The Gibco BRL enzyme is preferred.

With regard to PCR reactions, a wide variety of polymerases are suitable. These enzymes should exhibit thermostable activity, high fidelity and high processivity. Such enzymes are available from Boehringer Mannheim (Cat. Nos. 1146 165, 1146 173, 1418 432 and 1435 094) and Perkin Elmer (Cat. Nos. N801-0060 and N808-1012). The preferred enzyme is that made by Perkin Elmer.

To determine the optimal RT procedure, different reaction times were tested. Surprisingly, by reducing the time from the traditional 45-60 minutes to 15 minutes, yields were increased. In addition, the 15-minute reaction was characterized for accuracy by sequence analysis and found to have a high degree of fidelity. Michael *et al.*, *J. AIDS* 6(10):1073-1085 (1993). Another unique aspect involves the implementation of pIC/T7 polymerase-generated RNA control transcripts at the RT step, thus providing a positive control for the highly sensitive reverse transcription

reaction. A blank or "mock" RT reaction is also performed to monitor for the presence of contaminating DNA's

For use with the phosphorescent imaging system described below, probes must be labeled isotopically, preferably with a ß-emitter such as ^{32}P. Typically, probes are end-labeled using standard procedures. Probes for *gag*, ß-actin and ß-globin sequences are all distinct from the primers used to generate the PCR products. As mentioned, the general benefit of using a separate probe step, as opposed to detection via labeled primers, is that products are resolved independent of their molecular weight, thus providing a far greater degree of confidence in the results. In addition, the use of distinct probes hybridizing to the internal regions of the PCR product avoids the detection of spurious amplification products, an effect seen when the probes are identical to the amplification primers.

The adaptation of quantitative PCR to employ liquid rather than membrane hybridization was undertaken for several reason including speed, simplicity and efficiency. Typically, hybridization of oligonucleotide probes to target DNA is performed by immobilizing the target on a nitrocellulose or nylon matrix by virtue of hydrogen bonding. The approach requires that the target DNA be transferred from a gel to the matrix by capillary action and then denatured. Prehybridization and hybridization steps require up to 16 hours, largely because of molecular constraints on the target DNA resulting from the immobilization thereof. Overall, membrane hybridization procedures may take up to three days. This approach suffers from (i) loss of target, (ii) diminished efficiency of hybridization and (iii) excessive consumption of time, among other defects.

By way of contrast, liquid hybridization does not require immobilization of target DNA sequences on a matrix. Rather, the target DNA and probe are brought together in a liquid environment. The dynamics of the

hybridization reaction are far more favorable in liquid than on a matrix because there are no constraints placed on the conformation of the target *in solutio*. Consequently, the interaction of the target DNA with the probe is several orders of magnitude more efficient than matrix based assays, thereby significantly reducing the time needed for efficient hybridization (~15 min. total). Another major benefit of liquid hybridization is the reduction in post-hybridization handling. This is important in that one of the major sources of inaccuracy in PCR assays involves user error. By minimizing the number steps and the complexity of the reaction as whole, the opportunities for variation and mistake are similarly reduced.

The challenge in adapting quantitative PCR to employ liquid hybridization, however, was two-fold. First, it was not clear that hybridization chemistries would be found that provided the necessary efficiency and specificity. Second, it was unknown whether adequate product separation methods could be found even if the hybridization chemistries could be perfected. In order to address both of these concerns, the possibility of single-band detection was investigated. Using a unique, empirically-derived combination of primers and probes and optimized PCR and liquid hybridization chemistries, followed by a standard acrylamide mini-gel separation for about 15 min., rapid and accurate detection of a PCR product was achieved. By basing the assay on detection of single, well-defined band, the added benefit of improved confidence in the results also is achieved.

One of the important features of the instant invention is the use of the Molecular Dynamics PHOSPHOR IMAGER™ system. This system is described by Johnston *et al.*, *Electrophoresis* **11**:355-360 (1990). Briefly, phosphorimaging involves the use of photostimulable, phosphorescent imaging plates as an alternative to film for the recording and quantifying of autoradiographic images. These images are converted to a video signal

which is capable of storage on computer. Thus, data storage, retrieval and analysis capabilities are considerable.

As stated above, the amount of target nucleic acid is determined on a per cell basis. Because HIV-1 replicates in cells, attempts to quantitate HIV-1 are most meaningful if expressed as copies of virus per cell. This is a biologically relevant measure as opposed to an arbitrary molar amount which, incidentally, is very hard to determine on an absolute basis when levels are very low. Most assays cannot accurately determine target nucleic acid per cell equivalent and, therefore, cannot offer a biological context for whatever quantitative results are achieved. Clearly, cell based equivalency cannot be used with acellular samples such as plasma. By convention, the amount of viral nucleic acid in a plasma sample is determined on a per ml basis.

The approach of the present invention could be adapted for use with other target sequences, both endogenous and exogenous to a target organism. For example, the present method could be modified to identify and quantitate mRNA species from a cellular gene whose levels were implicated in development of a disease state. Alternatively, primers could be designed that would amplify nucleic acids from other infectious agents. The modifications described, however, would require extensive experimentation into (i) which target sequences should be used as models for both probes and primers, (ii) what amplification conditions would lead to quantitatable results and (iii) what hybridization conditions would yield the most unambiguous data. This is by no means a routine or predictable undertaking and some systems may not be successful as others in particular parameters.

The following examples are illustrative of the practice of the invention but should not be read as limiting the scope thereof.

EXAMPLE 1: ISOLATION OF RNA AND DNA FROM PATIENT SAMPLES

I. Isolation of RNA and DNA from Peripheral Blood Mononuclear Cells

A. Preparation of Peripheral Blood Mononuclear Cell Samples

Blood samples are collected from patients in acid-dextrose anticoagulant venipuncture tubes (Fisher Scientific). Approximately 80-100 ml of blood is drawn from each patient and processed within four hours of collection. Processing begins by separating cells from plasma with a 10 min. centrifugation at 500g. Plasma is transferred to a clean tube and further clarified by centrifugation for 30 min. at 500g. Clarified plasma is aliquoted at 0.5 ml and stored at -80°C. A suitable amount of the cell fraction is added to Ficoll-Hypaque media (Sigma) and the mixture is centrifuged for 30 min. at 500g. Aliquots of 5 X 10^6 cells are stored at -180°C.

Each peripheral blood mononuclear cell sample was prepared for nucleic acid isolation as follows. The cell samples were removed from liquid nitrogen storage and thawed rapidly in a 37°C water bath. Approximately 1 ml of cell sample was diluted with 5 ml of RPMI (Advanced Biotechnologies, Inc.) containing 10% fetal calf serum, and the diluted sample was centrifuged for 10 minutes at 1K RPM in a Beckman GPR tabletop centrifuge at room temperature.

After decanting the supernate, the pelleted cells were resuspended in 5 ml of room temperature 1X phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH to 7.4 with HCl). A 12 μl aliquot of the cell suspension was removed from the sample, and used to determine the numbers of viable and dead cells with a hemocytometer. The cell suspension was again pelleted by centrifuging for 5 minutes at 1.5K RPM at room temperature. After decanting the supernate, the cells were resuspended in 5 ml of room temperature 1x PBS. One ml aliquots of the resuspended cells were transferred to 4 ml Falcon test tubes.

B. Isolation of RNA from Peripheral Blood Mononuclear Cells

Falcon test tubes containing 1 ml aliquots of cell suspension were centrifuged for 5 minutes at 1500 RPM in a Beckman GPR tabletop centrifuge at room temperature. The supernate was decanted, and the cells were resuspended in the appropriate volume of RNA STAT-60 (Tel-Test B, Inc., Friendswood, TX). One milliliter of RNA STAT-60 was added per 5-10 x 10^6 cells, or 800 μl of RNA STAT-60 were added if the tube contained fewer than 5 x 10^6 cells. The cells were lysed by repetitive pipetting.

After allowing lysates to incubate at room temperature for 5 minutes, the lysates were transferred to 1.5 ml microfuge tubes. To extract the lysates, 200 μl of chloroform were added per 1 ml of RNA STAT-60. The tubes were vortexed vigorously for 15 seconds, and the samples were allowed to stand at room temperature for 2 to 3 minutes. The samples were centrifuged for 15 minutes at 12,000 g in an Eppendorf 5415 C centrifuge at 4°C. The upper aqueous phase containing RNA was transferred to a fresh tube. Next, 800 μl of DNA STAT-60 were added to the organic phase and treated as described below under section "C."

Five hundred microliters of isopropanol were added per 1 ml of RNA STAT-60 that had been added previously. The tubes were vortexed, and the samples were incubated for 5 to 10 minutes at room temperature. Samples were centrifuged for 10 minutes at 12,000 g at 4°C. After decanting the supernate, precipitates were washed with 500 μl of cold 75% ethanol. Samples were centrifuged for 5 minutes at 7,500 g at 4°C. After decanting the supernate, samples were dried briefly by air-drying or in a vacuum (5 to 10 minutes). (A Speed-Vac should not be used to dry the samples.) After adding 100 μl of diethylpyrocarbonate (DEPC)-treated water to the pellets, samples were heated to 95°C for 5 minutes. The samples

were then vortexed vigorously to resuspend RNA. After repeating the 5 minute incubation at 95°C, samples were vortexed and chilled on ice for 5 minutes.

In order to inhibit RNase and to remove contaminating DNA, the following were added to each chilled RNA sample: 1 μl of 1.0 M dithiothreitol, 2.5 μl of RNasin (100 units; Promega or BRL), 2.5 μl of 100 mM Tris-HCL containing 40 mM $MgCl_2$ (pH 7.4), and 5.0 μl of DNase I (50 units). After incubating the mixtures at 37°C for 1 hour, samples were heated to 95°C for 15 minutes to inactivate DNase I. The samples were chilled on ice, and 3 μl of RNasin (120 units) were added to each sample. At this point, the RNA samples were ready for the reverse transcriptase reaction. RNA samples were stored at -80°C.

C. Isolation of DNA from Peripheral Blood Mononuclear Cells

Tubes containing the DNA STAT-60/chloroform mixture resulting from the RNA procedure described above were vigorously shaken for 15 seconds, and allowed to stand at room temperature for 2 to 3 minutes. Samples were centrifuged at 12,000g for 15 minutes at 4°C, resulting in the formation of a lower organic phase and an upper aqueous phase, which contained DNA. If necessary, the chloroform extraction was repeated.

Each aqueous phase was transferred to a fresh tube, and mixed with isopropanol (500 μl of isopropanol per 1 ml of DNA STAT-60). The samples were allowed to stand at room temperature for 5 to 10 minutes and then centrifuged at 12,000g for 10 minutes at 4°C. After removing the supernate, the DNA pellets were washed once with 1 ml of 75% ethanol per 1 ml of the DNA STAT-60 that had been used in the previous step. After a 5 minute centrifugation at 7,500g and 4°C, the pellet was dried briefly by air drying or vacuum (5 to 10 minutes). (A Speed-Vac should not be used to dry the DNA pellets.) DNA pellets were dissolved in 100 μl of DEPC-treated

water, and the tubes were vortexed vigorously. DNA samples were then incubated for 10 to 15 minutes at 55 to 60°C and repeatedly vortexed. At this point, the DNA samples were ready for the polymerase chain reaction. DNA samples were stored at -80°C.

II. Isolation of RNA and DNA from Patient Plasma Samples

A. Preparation of RNA from Patient Plasma Samples

One ml of plasma is thawed by manual agitation in a 37°C water bath and placed on ice. One ml of PBS containing a final concentration of 5 μg bovine serum albumin (BSA; Sigma) is added to the plasma. The mixture is centrifuged for 30 min. at 12,000g and 4°C. The supernatant is removed gently so as to leave the viral pellet undisturbed. The viral pellet is resuspended in 800 μl of Tri-reagent (Molecular Research Center, Cincinnati, OH). Next, 160 μl of chloroform (Sigma) is added followed by mixing. After sitting at room temperature for 3 min., the mixture is centrifuged briefly at 12,000g at room temperature. The upper phase is removed to a fresh tube. A second addition of 160 μl of chloroform is added followed by mixing, centrifugation and removal of the upper phase as above. Two μl of 4M sodium acetate and 200 μl of 2-propanol (Sigma) are added to the removed upper phase, mixed and the tube placed at -20°C for 30 min. The tube is then centrifuged at 12,000g at 4°C for 15 min. The supernatant is removed and the pellet is washed with one ml of cold 100% ethanol. The pellet is the air dried for 15 min. after which the pellet is resuspended in 50 μl of RNA suspension buffer (10 units RNAsin and 150 μl of DTT in 3 ml of sterile water) and stored at -70°C.

EXAMPLE 2: PREPARATION OF DNA TEST SAMPLES

I. Preparation of DNA Test Samples Using Reverse Transcription

Reverse transcription reactions were performed in a total reaction volume of 20 μl. Reaction mixtures contained: 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 20 mM dithiothreitol, 0.5 mM each of dATP, dGTP, dCTP, and dTTP, 10 Units/μl reverse transcriptase, 1 Unit/ml RNasin, 0.5 μM 3' GAG primer (CAT CCA TCC TAT TTG TTC CTG AAG G, SEQ ID NO:1) or 0.5 μM 3' actin primer (CAT CTC TTG CTC GAA GTC CA, SEQ ID NO:2), and less than 5 μg of RNA template. Eight micrograms of RNA template were used for the reverse transcription of GAG RNA, and 4 μg of RNA template were used for the reverse transcription of actin RNA. Eight micrograms of template RNA were used for the corresponding pIC-derived standards. Reaction mixtures were placed in a thermocycler, and heated for 10 minutes at 70°C. After chilling the samples to 4°C, reaction tubes were centrifuged and placed on ice.

The following components were then added to each chilled reaction mixture: 4.0 μl of 5x reaction buffer (250 mM Tris-HCl at pH 8.3, 375 mM KCl and 15 mM $MgCl_2$), 2.0 μl of 0.1 M dithiothreitol, 0.5 μl of RNasin (40 Units/μl), 1.0 μl of reverse transcriptase (200 Units/μl), and 0.4 μl of a dNTP mix containing 25.0 mM each of dATP, dCTP, dGTP, and dTTP. The tubes were again placed in a thermocycler, and incubated at 45°C for 15 minutes. After incubating the tubes at 95°C for 10 minutes to inactivate reverse transcriptase, the tubes were chilled to 4°C.

II. Preparation of DNA Test Samples Using the Polymerase Chain Reaction

A. Linearization of pIC Plasmids

The pIC plasmids were prepared as follows. pIC-glo was linearized with *Bam* HI and pIC-*gag* with *Xba* I. Each

digestion mixture contained 50 μg of plasmid, 10 μl of the appropriate 10x buffer, 100 Units of the appropriate enzyme, and distilled water to a final volume of 100 μl. Plasmids were digested at 37°C for 1 hour. Agarose gel electrophoresis was used to verify that the plasmids were completely digested. Linearized plasmid DNA was purified by extraction with phenol:chloroform, followed by ethanol precipitation. Precipitated plasmid DNA was lyophilized, and then resuspended in 50 μl TE buffer (10 mM Tris-HCl at pH 8.3, 1mM EDTA at pH 8.0). Linearized pIC plasmids were stored at 4°C.

B. pIC Dilution Calculations

The following formula was used to calculate the concentration of a pIC plasmid in terms of gram plasmid/copy: molecular weight (g/mol. nt.) X length (nt.) X $1/6.0223X10^{23}$ mol./copy. For RNA, the molecular weight is 341 gram/mol. nt, while for DNA, the molecular weight is 648 gram/mol. nt. The plasmid, pIC-glo (globin DNA/268 bp into pBluescript® II KS-, *Sma* I), contains 3403 nucleotides. The plasmid, pIC-*gag* (genomic DNA/1266 bp into pGEM®-3Z, *Xba* I), contains 4009 nucleotides. The pIC-derived RNA (*gag* RNA synthesis: pIC-*gag* DNA, *Xba* I, T7 RNA polymerase; standard conditions) contains 1300 nucleotides. Plasmid pIC-glo was diluted to obtain $5x10^4$, $1x10^4$, $5x10^3$, $1x10^3$, $5x10^2$, or $1x10^2$ copies of plasmid per polyacrylamide gel well. Plasmid pIC-*gag* was diluted to obtain $1x10^3$, $5x10^2$, $1x10^2$, 50, 10, or 5 copies of plasmid per polyacrylamide gel well. pIC-*gag*-derived RNA was diluted to obtain $1x10^4$, $5x10^3$, $1x10^3$, $5x10^2$, $1x10^2$, or 10 copies per polyacrylamide gel well. A solution of sterile water and 4 μg/ml carrier tRNA was used for each initial dilution. Subsequent dilutions were performed using a solution of sterile water containing 5 μM dithiothreitol, 1 Unit/μl RNasin, and 4 μg/ml of carrier tRNA.

C. The Polymerase Chain Reaction

Reaction tubes for the polymerase chain reaction (PCR) were prepared by adding either 30 μl of sample

template patient DNA (the final concentration of DNA was less than 10 μg/tube), 30 μl of stock pIC DNA (prepared by diluting 10 μl of linearized pIC DNA with 20 μl of distilled water), 30 μl of template DNA obtained from a reverse transcriptase reaction (*i.e.*, the entire 20 μl reverse transcriptase reaction mixture plus 10 μl of distilled water), or 30 μl of diluted beta-globin template (prepared by diluting 2 μl of beta-globin template from a working stock dilution series from 5 X 10^4 to 1 X 10^2 copies of plasmid per ml with 28 μl of distilled water). A 70 μl volume of a PCR reaction mix was added to each tube. Each 70 μl volume of the PCR reaction mix contained 47.7 μl of distilled water, 10.0 μl of 10x buffer (500 mM KCl, 100 mM Tris-HCl at pH 8.3, 15 mM MgCl2 and 0.01% (w/v) gelatin), 5.0 μl of 5' primer (20 μM), 5.0 μl of 3' primer (20 μM), 1.0 μl of 20 mM dUTP, 0.8 μl of dNTP solution (25 mM each of dATP, dCTP, dTTP and dGTP), and 0.5 μl of *Taq* polymerase (5 U/μl). One microliter of uracil N-glycosylase was then added to each tube, and the solutions were mixed.

The polymerase chain reaction was performed with the "OO" primers, GAG-3 (CAATGAGGAAGCTGCAGAATGGGATAG, SEQ ID NO:3; nucleotides 1412-1438 of MN strain, GENBANK) and GAG-6 (CATCCATCCTATTTGTTCCTGAAGG, SEQ ID NO:4; nucleotides 1547-1523 of MN strain, GENBANK), using the following thermal cycler conditions:

Thermo-cycle file:	95°C denature	30 sec
	55°C anneal	30 sec
	72°C extension	3 min
Time delay file:	72°C extension	10 min
Soak file:	4°C soak	indefinite
Cycle number:	25 - DNA	28 - RNA

The polymerase chain reaction was performed with the globin primers, Globin-5 (GAAGAGCCAAGGACAGGTAC, SEQ ID NO:5; nucleotides -195 to -176 of human ß-globin gene, GENBANK) and Globin-3 (CAACTTCATCCACGTTCACC, SEQ ID NO:6; nucleotides 73 to 54 of human ß-globin gene, GENBANK), using the following thermal cycler conditions:

Thermo-cycle file:	95°C denature	30 sec
	50°C anneal	30 sec
	72°C extension	3 min
Time delay file:	72°C extension	10 min
Soak file:	4°C soak	indefinite
Cycle number:	22	

The polymerase chain reaction was performed with the actin primers, act5 (ATCATGTTTGAGACCTTCAA, SEQ ID NO:7; nucleotides 1854 to 1873 of human ß-actin gene, GENBANK) and act3 (CATCTCTTGCTCGAAGTCCA, SEQ ID NO:8; nucleotides 2170 to 2151 of human ß-actin gene, GENBANK), using the following thermal cycler conditions:

Thermo-cycle file:	94°C denature	30 sec
	45°C anneal	30 sec
	72°C extension	3 min
Time delay file:	72°C extension	10 min
Soak file:	4°C soak	indefinite
Cycle number:	22	

The products of the various polymerase chain reactions were analyzed using the liquid hybridization technique, as described below.

EXAMPLE 3: ANALYSIS OF PCR PRODUCTS

I. Preparation of Radiolabeled Oligonucleotide Probes

Oligonucleotide probes were synthesized with 5' hydroxyl groups using trityl synthesis and an Applied Biosytems DNA Synthesiszer. The following oligonucleotide probes were used in liquid hybridization analysis:

(a) ATGAGAGAACCAAGGGGAAGTGACATAGCA, SEQ ID NO:9 (GPR-5; nucleotides 1479 to 1506 of MN strain, GENBANK) for detection of GAG sequences,

(b) AAGTCAGGGCAGAGCCATCTATTGCTTACA, SEQ ID NO:10 (GLO-probe; nucleotides -77 to -48

of human ß-globin gene, GENBANK) for detection of globin sequences, and

(c) GACCTGGCTGGCCCGGACCTGACTGACTAC, SEQ ID NO:11 (ACT-probe; nucleotides 2026 to 2055 of human ß-actin gene, GENBANK) for detection of actin sequences.

Oligonucleotide probes were labeled with ^{32}P using a standard T4 polynucleotide kinase technique (Boehringer Mannheim or New England Biolabs) and radiolabeled probes were purified using G-25 spin columns according to the manufacturer's instructions (5 Prime → 3 Prime, Inc., Boulder, CO).

II. Liquid Hybridization Analysis

An appropriate volume of probe mix was prepared depending upon the number of reactions to be hybridized. The probe mix for one reaction was prepared by diluting the radiolabeled probe in distilled water, transferring 5.0×10^4 cpm of the diluted probe to a microfuge tube, and adding 1.1x OH buffer (667 µl of 1M NaCl, 888 µl of 0.5M EDTA, pH 8.44, in 5 ml of distilled water) to bring the total volume to 11 µl. A 10 µl aliquot of the probe mix was transferred to a 0.5 ml microfuge tube, and 30 µl of the test PCR product was added to the tube. After mixing the contents of the reaction tube, the tube was centrifuged to pellet the mixture. The reaction mixture was incubated at 94°C for 5 minutes, and then at 55°C for 10 minutes. After allowing the mixture to briefly cool at room temperature, 4 µl of 5x DNA loading buffer (5 ml 10XTBE, 0.5 ml 1% bromophenol blue, 3 ml 100% glycerol and 1.5 ml H_2O) were added to the mixture. The contents of the reaction tube were mixed, and the tube was centrifuged to pellet the mixture.

The liquid hybridization reaction was analyzed by fractionating the reaction mixture on a 10% polyacrylamide gel. The gel was pre-run for 15 to 20 minutes at 200 volts in 1x TBE (89 mM Tris base, 89 mM

boric acid, 1 mM EDTA at pH 8.0). Ten microliters of reaction mixture were loaded per well, and the gel was run for approximately 30 min at 200 volts. The gel was then removed from the electrophoresis apparatus, covered in plastic wrap, placed on the imaging plate and inserted into a PHOSPHOR IMAGER™ cassette, and exposed for 1 hour. Plates are erased prior to use by exposure to visible light.

The results were analyzed using a Molecular Dynamics PHOSPHOR IMAGER™. This instrument scans commercially available phosphorescent imaging plates with a 10 mW helium-neon laser by means of a galvanometer-controlled mirror. The Gaussian beam is focused on an 88 μm spot ($1/e^2$ point) at the plane of the imaging plate where the laser power is about 7 mW. The beam sweeps across a 35 cm wide plate in about 200 msec, or 50 μsec per 88 μm pixel. These parameters permit 80% of image information on the plate to be read. As the plate is scanned, the luminescence at 390 nm is collected by a fiber optic coupler and channeled to a low dark current, high dynamic range, Hamamatsu photomultiplier tube. Filters placed before the photomultiplier tube achieve 10^{15} attenuation of the stimulating laser light. The positionally-defined data stream passes through a log amplifier and analog-to-digital (AD) converter, is five times oversampled at 100 kHz, integrated, and stored as 16 bit data on magnetic hard disk. The AD converter is a state-of-the-art 16 bit sigma-delta device requiring neither antialiasing filters nor external sample and hold circuitry. All data acquisition and intermediate processing are controlled by two Motorola 56001 Digital Signal Processors guaranteeing 16 bit accuracy over five orders of dynamic range. All scanner operations, data display and analysis are performed using Molecular Dynamics ImageQuant software operating on an Intel 80286 or 80386 microprocessor.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: U.S. Department of the Army
(B) STREET: Medical Research and Development Command
(C) CITY: Fort Detrick
(D) STATE OR PROVINCE: Maryland
(E) COUNTRY: U.S.
(F) POSTAL CODE: 21702

(ii) TITLE OF INVENTION: HIGH THROUGH-PUT QUANTITATIVE POLYMERASE CHAIN REACTION FOR HIV CLINICAL SPECIMENS

(iii) NUMBER OF SEQUENCES: 11

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(v) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: PCT
(B) FILING DATE:

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/154,416
(B) FILING DATE: 19-NOV-1993

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CATCCATCCT ATTTGTTCCT GAAGG 25

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CATCTCTTGC TCGAAGTCCA 20

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CAATGAGGAA GCTGCAGAAT GGGATAG 27

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CATCCATCCT ATTTGTTCCT GAAGG 25

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GAAGAGCCAA GGACAGGTAC 20

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CAACTTCATC CACGTTCACC 20

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ATCATGTTTG AGACCTTCAA 20

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CATCTCTTGC TCGAAGTCCA 20

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ATGAGAGAAC CAAGGGGAAG TGACATAGCA 30

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

AAGTCAGGGC AGAGCCATCT ATTGCTTACA 30

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GACCTGGCTG GCCCGGACCT GACTGACTAC 30

WE CLAIM:

1. A method for the quantitative detection of an HIV DNA in a tissue sample, comprising the steps of:

(i) extracting said HIV DNA from said sample;

(ii) contacting said HIV DNA with

(a) at least four different nucleotide triphosphates,

(b) a first primer that hybridizes to the *gag* region of said HIV DNA, and

(c) an enzyme with polynucleotide synthetic activity,

under conditions suitable for the hybridization and extension of said first primer by said enzyme, whereby a first DNA product is synthesized with said HIV DNA as a template therefor, such that a duplex molecule is formed;

(iii) denaturing said duplex to release said first DNA product from said HIV DNA;

(iv) contacting said first DNA product with a reaction mixture comprising

(a) at least four different nucleotide triphosphates,

(b) a second primer that hybridizes to said DNA product, and

(c) an enzyme with polynucleotide synthetic activity,

under conditions suitable for the hybridization and extension of said second primer by said enzyme, whereby a second DNA product is synthesized with said first DNA product as a template therefor, such that a duplex is formed;

(v) denaturing said second DNA product from said first product;

(vi) repeating steps ii-v for a sufficient number of times to achieve linear production of said first and second DNA products;

(vii) contacting said first and second DNA products with a first oligonucleotide probe that is labeled and that hybridizes to said first and second products, wherein said contacting is performed in a liquid environment under conditions suitable for hybridization;

(viii) detecting hybridization of said first probe with said first and second DNA products;

(ix) concurrently performing steps ii-viii upon a known amount of a *gag* control DNA in a separate reaction; and

(x) comparing the amount of first and second products generated from said DNA with the amount of products generated from said *gag* control DNA,

whereby the amount of HIV DNA in said sample is quantitated.

2. The method of claim 1, wherein said sample is a biological sample selected from the group consisting of human whole blood and human peripheral blood lymphocytes.

3. The method of claim 1, wherein said sample is a standardized volume of human plasma.

4. The method of claim 1, wherein said detecting is performed using a phosphor imaging system.

5. The method of claim 4, wherein the dynamic range of said method is 5-6 logs.

6. The method of claim 1, wherein said *gag* control DNA is pIC-*gag*.

7. The method of claim 6, wherein said first primer is GAG-3 and said second primer is GAG-6.

8. The method of claim 7, wherein said first probe is GPR-5.

9. The method of claim 2, wherein step ii is performed in the presence of a third primer that hybridizes to a second DNA derived from a cell in said sample, step iii further comprises synthesizing a third

DNA product, wherein said second DNA is a template for said synthesis, step iv is performed in the presence of a fourth primer that hybridizes to said third DNA product, step v further comprises synthesizing a fourth DNA product, step vii is performed in the presence of a second oligonucleotide probe that hybridizes to said third and fourth DNA products, step viii further detects the hybridization of said second probe with said third and fourth DNA products, step ix further concurrently performs steps ii-viii on a second control DNA that hybridizes to said third and fourth primers and step x further compares the amount of said third and fourth DNA products with the amount of said first and second DNA products, whereby the amount of said HIV DNA per cell of said sample is determined.

10. The method of claim 9, wherein said second DNA is a ß-globin DNA.

11. The method of claim 10, wherein said second control DNA comprises pIC-glo.

12. The method of claim 11, wherein said third primer is Globin-5 and said fourth primer is Globin-3.

13. The method of claim 12, wherein said second probe is GLO-probe.

14. A method for the quantitative detection of an HIV RNA in a tissue sample, comprising the steps of:

(i) extracting said RNA from said sample;

(ii) contacting said RNA with

(a) at least four different nucleotide triphosphates,

(b) a sample reverse transcriptase primer that hybridizes to said RNA, and

(c) an enzyme with reverse transcriptase activity,

under conditions suitable for the hybridization and extension of said sample reverse transcriptase primer by said enzyme, whereby a complementary DNA product is synthesized, such that a duplex is formed.

(iii) contacting said cDNA with

(a) at least four different nucleotide triphosphates,

(b) a first primer that hybridizes to said RNA, and

(c) an enzyme with polynucleotide synthetic activity,

under conditions suitable for the hybridization and extension of said first primer by said enzyme, whereby a first DNA product is synthesized with said cDNA as a template therefor, such that a duplex molecule is formed;

(iv) denaturing said duplex to release said first DNA product from said cDNA;

(v) contacting said first DNA product with a reaction mixture comprising

(a) at least four different nucleotide triphosphates,

(b) a second primer that hybridizes to said first DNA product, and

(c) an enzyme with polynucleotide synthetic activity,

under conditions suitable for the hybridization and extension of said second primer by said enzyme, whereby a second DNA product is synthesized with said first DNA product as a template therefor, such that a duplex is formed;

(vi) denaturing said second DNA product from said first DNA product;

(vii) repeating steps ii-v for a sufficient number of times to achieve linear production of said first and second DNA products;

(viii) contacting said first and second DNA products with a first oligonucleotide probe that is labeled and that hybridizes to said first and second DNA products, wherein said contacting is performed in a liquid environment under conditions suitable for hybridization;

(ix) detecting hybridization of said first probe with said first and second DNA products;

(x) concurrently performing steps ii-ix upon a known amount of a *gag* control RNA in a separate reaction; and

(xi) comparing the amount of first and second DNA products generated from said RNA with the amount of products generated from said *gag* control RNA,

whereby the amount of HIV RNA in said sample is quantitated.

15. The method of claim 14, wherein said sample is a biological sample selected from the group consisting of human whole blood and human peripheral blood lymphocytes.

16. The method of claim 14, wherein said sample is a standardized volume of human plasma.

17. The method of claim 14, wherein said detecting is performed using a phosphor imaging system.

18. The method of claim 14, wherein the dynamic range of said method is 4-5 logs.

19. The method of claim 14, wherein said *gag* control RNA is generated by treating pIC-*gag* with T7 RNA polymerase.

20. The method of claim 19, wherein said first primer is GAG-3 and said second primer is GAG-6.

21. The method of claim 20, wherein said first probe is GPR-5.

22. The method of claim 14, wherein step iii is performed in the presence of a third primer that hybridizes to a DNA derived from a cell in said sample, step iv further comprises synthesizing a third DNA product, wherein said DNA is a template for said synthesis, step v is performed in the presence of a fourth primer that hybridizes to said third DNA product, step vi further comprises synthesizing a fourth DNA product, step viii is performed in the presence of a second oligonucleotide probe that hybridizes to said third and fourth DNA products, step ix further detects

the hybridization of said second probe with said third and fourth DNA products, step x further concurrently performs steps iii-ix on a control DNA that hybridizes to said third and fourth primers and step xi further compares the amount of said third and fourth DNA products with the amount of said first and second DNA products, whereby the amount of said HIV RNA per cell of said sample is determined.

23. The method of claim 22, wherein said DNA is a ß-globin DNA.

24. The method of claim 23, wherein said control DNA comprises pIC-glo.

25. The method of claim 24, wherein said third primer is Globin-5 and said fourth primer is Globin-3.

26. The method of claim 25, wherein said second probe is GLO-probe.

27. A kit for the quantitative detection of an HIV nucleic acid in a tissue sample, comprising

(i) control DNA's containing HIV *gag* and human ß-globin nucleotide sequences;

(ii) primers derived from HIV *gag*, human ß-globin and human ß-actin nucleotide sequences;

(iii) oligonucleotide probes that hybridize to HIV-1 *gag*, human ß-globin and human ß-actin nucleotide sequences bounded by and exclusive of the corresponding primers of ii; and

(vi) a liquid hybridization chamber containing hybridization buffer.

28. The kit of claim 26 further comprising

(i) dNTP's;

(ii) polymerase chain reaction buffer; and

(iii) reverse transcriptase reaction buffer.

29. The kit of claim 27, wherein said control DNA's are pIC-*gag* and pIC-glo, said primers are GAG-3, GAG-6, Globin-5, Globin-3, act5 and act3 and said probes are GPR-5, GLO-probe and ACT-probe.

FIG. 1

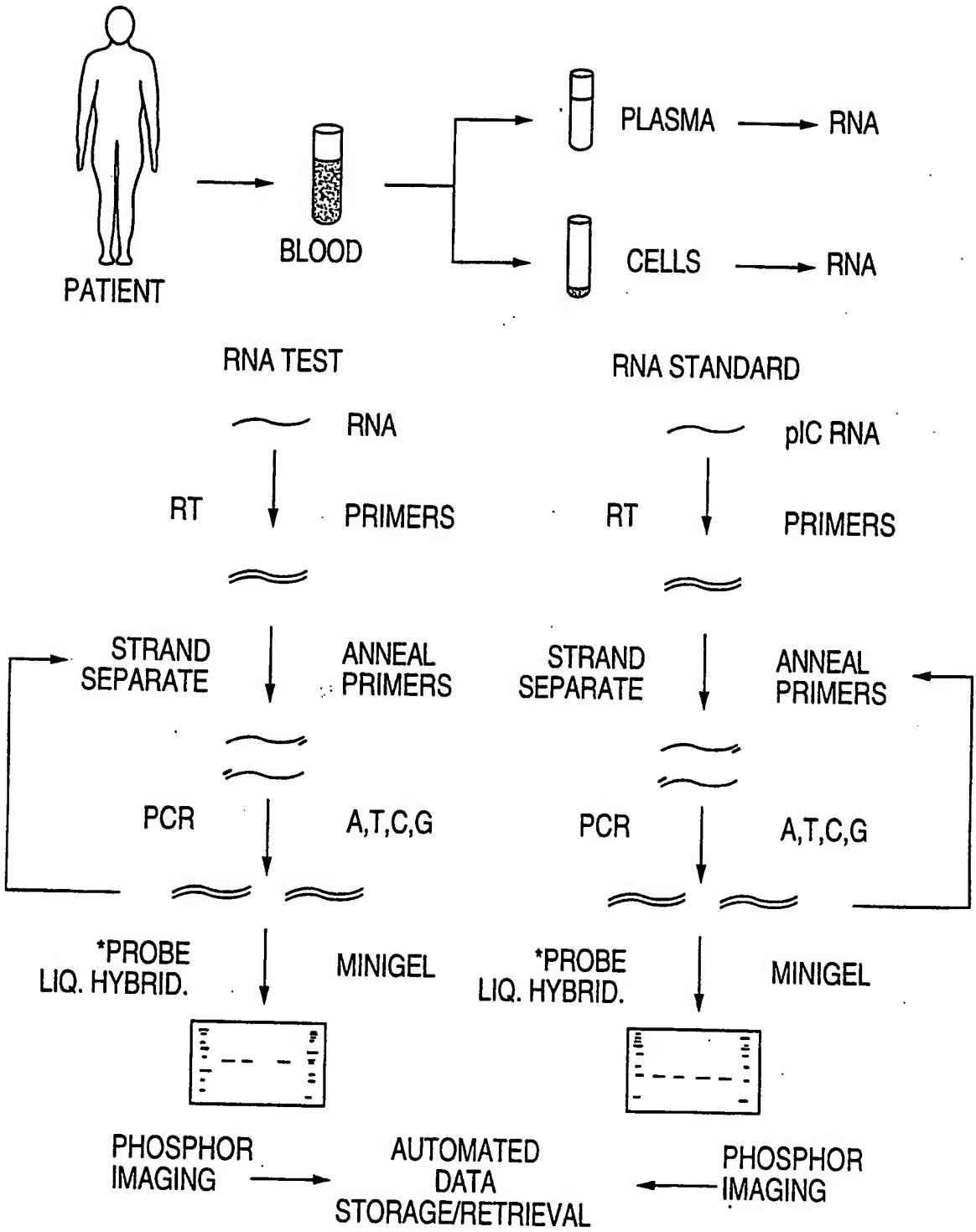
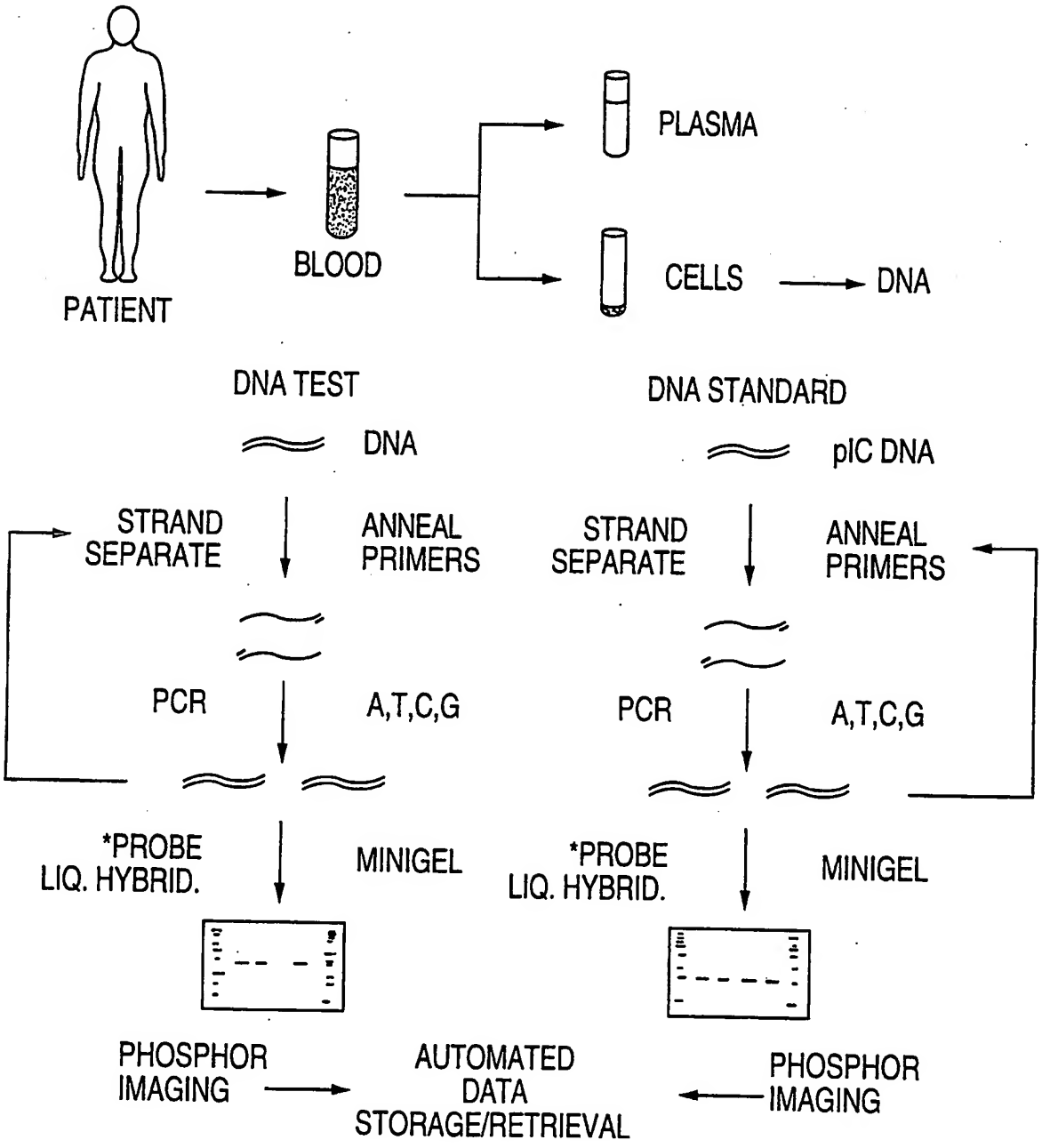

PATIENT
BLOOD
PLASMA
RNA
CELLS
RNA
RNA TEST
RNA
RT
PRIMERS
STRAND SEPARATE
ANNEAL PRIMERS
PCR
A,T,C,G
*PROBE LIQ. HYBRID.
MINIGEL
RNA STANDARD
pIC RNA
RT
PRIMERS
STRAND SEPARATE
ANNEAL PRIMERS
PCR
A,T,C,G
*PROBE LIQ. HYBRID.
MINIGEL
PHOSPHOR IMAGING
AUTOMATED DATA STORAGE/RETRIEVAL
PHOSPHOR IMAGING

FIG. 2


PATIENT
BLOOD
PLASMA
CELLS
DNA
DNA TEST
DNA
STRAND SEPARATE
ANNEAL PRIMERS
PCR
A,T,C,G
*PROBE LIQ. HYBRID.
MINIGEL
DNA STANDARD
pIC DNA
STRAND SEPARATE
ANNEAL PRIMERS
PCR
A,T,C,G
*PROBE LIQ. HYBRID.
MINIGEL
PHOSPHOR IMAGING
AUTOMATED DATA STORAGE/RETRIEVAL
PHOSPHOR IMAGING

INTERNATIONAL SEARCH REPORT

In. nal Application No
PCT/US 94/13514

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68 C12Q1/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	J. VIROL., vol.66, no.1, January 1992, AM. SOC. MICROBIOL. , BALTIMORE, US; pages 310 - 316 N.L. MICHAEL ET AL. 'Viral DNA and mRNA expression correlate with the stage of human immunodeficiency virus (HIV) type 1 infection in humans: Evidence for viral replication in all stages of HIV disease' cited in the application see page 310, right column, line 21 - page 312, right column, line 5 --- -/--	1-21

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
10 March 1995	20 -03- 1995

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+ 31-70) 340-3016	Hornig, H

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	AIDS RESEARCH AND HUMAN RETROVIRUSES, vol.9,SUPL.1, October 1993, MARY ANN LIEBERT, INC. PUBLISHERS, NEW YORK,US; page S80 M. ROBB ET AL. 'Development of a marker for HIV burden in pediatric patients using quantitytive RNA and DNA PCR of PBMC and plasma' 5th Annual meeting of the national coorperative vaccine development groups for AIDS on advances in AIDS vaccine development, Chantilly, Virginaia, USA, August 30-September 3, 1992; see abstract ---	1-3, 14-16
Y	MOLECULAR AND CELLULAR PROBES, vol.7, no.5, October 1993, ACADEMIC PRESS, LONDON, GB; pages 361 - 371 J. BÖNI AND J. SCHUPBACH 'Sensitive and quantitative detection of PCR-amplified HIV-1 DNA products by an enzyme linked immunoassay following solution hybridization with two differently labelled oligonucleotide probes' see page 362, right column, paragraph 3 - page 363, right column, paragraph 1 ---	1-3, 5-16, 18-21
Y	VOX SANGUINIS, vol.61, no.1, August 1991, S. KARGER AG, BASEL CH; pages 24 - 29 S.M. BRUISTEN ET AL. 'Enhanced detection of HIV-1 sequences using polymerase chain reaction and a liquid hybridization technique' see page 25, left column, line 6 - right column, line 11 ---	1-21
Y	J. CLIN. MICROBIOL., vol.27, no.11, November 1989, AM. SOC. MICROBIOL., WASHINGTON, DC,US; pages 2570 - 2573 W.F. CARMANI AND C. WILLIAMSON 'Detection of enzymatically amplified human immunodeficiency virus DNA by oligonucleotide solution hybridization and by incorporation of radiolabeled deoxynucleotides' see page 2570, right column, line 4 - page 2571, right column, line 12 ---	1-21

1

-/--

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ELECTROPHORESIS, vol.11, no.5, 1990, VCH VERLAGSGESELLSCHAFT MBH, WEINHEIM, BRD; pages 355 - 360 R.F. JOHNSTON ET AL. 'Autoradiography using storage phosphor technology' cited in the application the whole document	1-29

1